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Effect of the Combination of Co-Culture System and Supplemented Protein Sources on the *In Vitro* Development of Bovine IVF Embryos

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ABSTRACT

The present study was conducted to investigate the effects of various co-culture systems and supplemented protein sources on the *in vitro* development of bovine IVF embryos. Bovine cumulus oocyte complexes(COCs) were matured and fertilized *in vitro*. Presumptive zygotes with cumulus cells were transferred to TCM-199 or CR1aa containing 10% FBS or 3mg/ml BSA, and cultured for 36~40 hr. After primary culture, cleaved embryos were co-cultured with cumulus cells(CC), bovine oviduct epithelial cells(BOEC) or Buffalo rat liver cells(BRLC) in TCM-199 or CR1aa supplemented with FBS or BSA respectively, for further 6 days. Cleavage rate increased with BSA($P<0.01$) in the both TCM-199(79%) or CR1aa(74%). When embryos were co-cultured with CC or BOEC in TCM-199, blastocyst development was enhanced with BSA(40% and 43%) compared to FBS(22% and 29%), whereas in CR1aa no difference observed between BSA(40% and 39%) and FBS(40% and 42%). When embryos were co-cultured with BRLC monolayer, FBS enhanced the blastocyst development($P<0.05$) compared to BSA in both TCM-199(41% vs 31%) and CR1aa(44% vs 37%). The result of the present study showed that the cleavage rate of bovine IVF embryos increased with BSA, The result also showed that BSA can enhance the development of IVF embryos in co-culture with CC or BOEC in TCM-199, suggesting the *in vitro* development is affected by the medium and supplemented protein sources in co-culture with somatic cells.

(Key words: Co-culture, Protein source, *In vitro* development, Bovine IVF embryo)

I. INTRODUCTION

In bovine the establishment of *in vitro* culture system is necessary for the production of IVF derived-offspring, the security of embryo needed to researches of cloning or gene transfer, and the production of cloned or transgenic animals. Conventional *in vitro* culture systems have proved inadequate for the culture of bovine embryos from early cleavage to the blastocyst stage. A block at the 8- to 16-cell stage was observed in these *in vitro* culture systems of bovine early embryos(Wright and Bondioli, 1981).

Various co-culture systems were employed to overcome the *in vitro* cell-block of bovine em-

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bryos using various types of somatic cells, such as oviduct epithelial cells(Eyestone and First, 1989; Ellington et al., 1990; Rorie et al., 1994; van Inzen et al., 1995; Edwards et al., 1997), cumulus cells(Goto et al., 1988; Aoyagi et al., 1990; Zhang et al., 1995), granulosa cells(Fukui and Ono, 1989; Goto et al., 1994), trophoblastic vesicles(Heyman et al., 1987; Aoyagi et al., 1990; Nakao and Nakatsuji, 1990) and Buffalo rat liver cells(Voelkel and Hu, 1992; Hernandez-Ledezma et al., 1993; Rehman et al., 1994a,b; van Inzen et al., 1995). These co-culture system mainly used the monolayer of the cells in the complex medium containing serum. Generally, TCM-199 supplemented with 10% FBS were used for co-culture of bovine embryos with somatic cells. Indeed, such complex tissue culture media were designed specifically for sustaining somatic cells *in vitro* rather than embryos. Furthermore, serum has been shown to cause perturbations in embryo morphology, metabolism, ultrastructure and in post-implantation development(Thompson et al., 1995).

On the other hand vesicle suspension of the somatic cells were employed for co-culture system instead of monolayer cells. At this time, it was suggested that BSA enhance the *in vitro* development of bovine embryos(Moore and Bondioli, 1993). Simple media were also used for co-culture of bovine embryos with somatic cells (Rorie et al., 1994).

The present study was conducted to investigate the effects of various co-culture systems and supplemented protein sources on the *in vitro* development of bovine IVF embryos. TCM-199 and CR1aa were employed for *in vitro* culture medium because these media were used for *in vitro* culture of bovine embryos in popularly. BSA and FBS were employed for supplement protein source for the same reason.

Ⅱ. MATERIAL AND METHODS

1. Oocyte Recovery and *In Vitro* Maturation(IVM)

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory in 0.9% saline at 25~30℃. Follicular oocytes were aspirated from 2 to 7 mm follicles with a 18G needle and a 10 ml syringe. Oocytes were washed several times in maturation medium, and oocytes with a complete cumulus and ooplasm with homogeneous appearance were introduced into 50㎕ droplets of maturation medium(10 oocytes/droplet), covered with mineral oil and cultured under an atmosphere of 5% CO_2 in air at 39℃ for 22~24 hr. The maturation medium was TCM-199 with Earle's salts and 25 mM Hepes (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum(FBS; Gibco-BRL), 0.02U/ml FSH(Sigma, St. Louis, MO), 1㎍/ml β17-estradiol(Sigma), 0.2 mM Na-pyruvate and 50㎍/ml gentamycin(Sigma).

2. *In Vitro* Fertilization

In vitro fertilization was performed using frozen semen from one bull. Motile spermatozoa were obtained using frozen-thawed semen overlaid onto a 45:90% percoll(Pharmacia, Uppsala, Sweden) density gradient and centrifuged for 10 min at 700×g. Supernatant was removed and sperm pellet was resuspended in 7 ml BSA-free BO medium(3) containing 10 mM caffeine (Sigma) and washed by centrifugation at 500×g for 5 min. Spermatozoa were resuspended in BSA-free BO medium containing 10 mM caffeine at a concentration of 4×10^6 spermatozoa/ml of medium. Fertilization droplets were made by 25㎕ of sperm suspension was added to each droplet(25㎕) of BO medium containing 6 mg/ml BSA(Fraction V; Sigma, A6003) and 20㎍/ml heparin(Sigma) to give a final concen-

tration of 2×10^6 spermatozoa/ml, 5 mM caffeine, 10μg/ml heparin and 3 mg/ml BSA. About 10 *in vitro* matured cumulus-oocyte complexes(COCs) were introduced to each fertilization droplet and were incubated for 12~20 hr in 5% CO_2 in humidified air at 39℃.

3. Preparation of Somatic Cell Co-culture

Cumulus cell(CC) culture were prepared by cumulus cells separated from embryos at 32~40 hr of culture(see below) were transferred to 50 μl droplets of TCM-199 or CR1aa supplemented with 10% FBS or 3 mg/ml BSA.

Bovine oviduct epithelial cell(BOEC) vesicle suspensions were prepared according to the previous study(Eyestone and First, 1989) with TCM-199 or CR1àa supplemented with 10% FBS or 3 mg/ml BSA. Briefly, oviductal cells were collected from bovine oviduct by scraping with a glass slide. Oviductal cells were transferred to a centrifuge tube with TCM-199 or CR1aa supplemented with 10% FBS or 3 mg/ml BSA. Cells were then washed in 4~5 changes of each medium. After washing, oviductal cells were resuspended in each *in vitro* culture medium to a ratio of 1:50, and 3 ml suspension were cultured into 50 ml culture flasks(Corning, NY) for 2 to 3 days. Droplets(50μl) of the cell suspension were prepared under mineral oil in ϕ35 mm dishes (Nunc, Roskilde, Denmark).

The Buffalo rat liver cell(BRLC) cultures were obtained frozen from ATCC(Rockville, MD). BRLC were thawed in a 37℃ water bath for 1~2 min and suspended in 7~8 ml TCM-199 containing 10% FBS, Na-pyruvate and gentamycin. The suspension was centrifuged at 500×g for 5 min and the supernatant discarded. The pelleted BRLC were resuspended in 5 ml media and placed into a 50 ml tissue culture flask at 39℃ in humidified 5% CO_2 in air for 5~7 days. Media replaced every 2~3 days. Confluent BRLC monolayer were dispersed using 0.05% trypsin-EDTA(Sigma) and centrifuged at 500× g for 5 min. The pelleted BRLC were resuspended in TCM-199 containing 10% FBS at a concentration of 5×10^4 cells/ml, and cultured in 50μl droplets under mineral oil in ϕ35 mm dish (Nunc) for 2~3 days producing confluent BRLC monolayers. Medium was replaced with each *in vitro* culture medium 3~4 hr in advance.

4. *In Vitro* Culture

After *in vitro* fertilization, presumptive zygotes with cumulus cells were transferred to TCM-199 or CR1aa containing Na-pyruvate, gentamycin and 10% FBS or 3 mg/ml BSA, and cultured under an atmosphere of 5% CO_2 in air at 39℃ for 36~40 hr. After culture, embryos were separated from CC by pipetting, and cleaved embryos were cultured with or without CC, BOEC or BRLC in 50μl droplets of TCM-199 or CR1aa supplemented with 10% FBS or 3 mg/ml BSA respectively, for further 6 days. For BOEC and BRLC co-culture, media were replenished every 2~3 days by replacing 50% of the total volume with fresh media.

5. Statistical Analysis

Data were analyzed by the SAS generalized liner model(GLM) program. Treatment means were compared using least significant difference(LSD) test.

Ⅲ. RESULTS

1. Cleavage Rate of IVF Oocytes

The proportions of initial cleavage of the IVF oocytes were significantly high($P<0.01$) with BSA(74.9 and 74.3%) than FBS(50.0 and 56.8%) in the both TCM-199 and CR1aa(Table 1). There was interaction between medium and protein source($P<0.05$).

Table 1. Effects of different protein sources and medium on the initial cleavage of IVF bovine embryo

Medium	Protein source	No. of oocytes inseminated	%(mean±SE) of oocytes cleaved
TCM-199	BSA	118	79.4±1.9[a]
	FBS	115	50.0±3.2[b]
CR1aa	BSA	111	74.3±1.6[a]
	FBS	111	56.8±1.8[b]

Total of 5 replicates.

[a,b]Values with different superscripts differ($P<0.01$).

2. *In Vitro* Development in Simple Culture

Developmental rate to the blastocyst stage of cleaved embryos was high with culture in CR1aa(24.5~26.4%) than in TCM-199(12.2~15.1%) regardless of protein sources, when embryos were cultured without somatic cells(Table 2). However, the proportion of morulae plus blastocysts in TCM-199 supplemented with BSA(36.8%) was not different from those in CR1aa(39.1 and 41.2%).

3. *In Vitro* Development in Co-culture with CC

When the embryos were co-cultured with CC in TCM-199, developmental rate to the blastocyst stage of embryos was significantly higher ($P<0.01$) with BSA(40.2%) than FBS(22.2%). Developmental rates to the blastocyst stage(39.5~40.2%), however, equally high and were not affected by the supplemented protein sources in CR1aa(Table 3). Similar trend was shown in the proportion of morulae plus blastocysts. There were interactions between medium and protein source in the proportions of blastocyst and morulae plus blastocysts($P<0.01$).

4. *In Vitro* Development in Co-culture with BOEC

When embryos were co-cultured with BOEC in TCM-199, developmental rate to the blastocyst stage of embryos was significantly higher ($P<0.05$) with BSA(43.2%) than FBS(29.4%). However, developmental rates to the blastocyst stage(38.6~42.4%) were equally high and were not affected by the supplemented protein sour-

Table 2. *In vitro* development of IVF bovine embryos in TCM-199 or CR1aa supplemented with different protein sources

Medium	Protein sources	No. of embryos cultured*	%(mean±SE) of embryos developed to		
			Morula	Blastocyst	M+Bl**
TCM-199	BSA	100	21.8±2.5	15.1±1.2[a]	36.8±2.6[a]
	FBS	96	17.7±3.6	12.2±1.7[a]	29.9±3.6[b]
CR1	BSA	101	16.7±4.6	24.5±2.0[b]	41.2±3.7[a]
	FBS	106	12.6±2.9	26.4±3.5[b]	39.1±1.6[a]

Total of 4 replicates.

* Two- to 8-cell stage embryos were used for *in vitro* culture.

** M: morula, Bl: blastocyst

[a,b]Values within columns with different superscripts differ($P<0.05$).

Table 3. *In vitro* development of IVF bovine embryos co-cultured with cumulus cells(CC) in TCM-199 or CR1aa supplemented with different protein sources

Medium	Protein sources	No. of embryos cultured*	%(mean±SE) of embryos developed to		
			Morula	Blastocyst	M+Bl**
TCM-199	BSA	104	13.5±0.7	40.2±1.4[a]	53.7±1.3[a]
	FBS	102	17.6±0.9	22.2±2.2[b]	39.8±1.7[b]
CR1	BSA	106	10.4±1.7	39.5±2.1[a]	49.9±1.4[a]
	FBS	108	8.3±0.4	40.2±2.9[a]	48.4±2.7[a]

Total of 4 replicates.

* Two- to 8-cell stage embryos were used for *in vitro* culture.

** M: morula, Bl: blastocyst

[a,b]Values within columns with different superscripts differ($P<0.05$).

Table 4. *In vitro* development of IVF bovine embryos co-cultured with bovine oviduct epithelial cells(BOEC) in TCM-199 or CR1aa supplemented with different protein sources

Medium	Protein sources	No. of embryos cultured*	%(mean±SE) of embryos developed to		
			Morula	Blastocyst	M+Bl**
TCM-199	BSA	108	10.7±4.2	43.2±2.7[a]	53.9±3.5[a]
	FBS	104	16.8±2.0	29.4±2.4[b]	46.2±0.7[b]
CR1	BSA	103	14.8±1.5	38.6±1.7[a]	53.3±1.5[a]
	FBS	102	16.7±2.2	42.4±1.8[a]	59.1±2.1[a]

Total of 4 replicates.

* Two- to 8-cell stage embryos were used for *in vitro* culture.

** M: morula, Bl: blastocyst

[a,b]Values within columns with different superscripts differ($P<0.05$).

ces in CR1aa(Table 4). Similar trend was shown in the proportion of morulae plus blastocysts. There were interactions between medium and protein source in the proportions of blastocyst and morulae plus blastocysts($P<0.01$).

5. *In Vitro* Development in Co-culture with BRLC

When embryos were co-cultured with BRLC in TCM-199 or CR1aa, development to the morula and blastocyst stage was affected by supplemented protein sources(Table 5). Developmental rates to the blastocyst stage were 40.9% and 44.4% in TCM-199 and CR1aa supplemented with FBS, respectively, whereas 30.5% and 37.4% in each medium supplemented with BSA. Similar trend was shown in the proportion of morulae plus blastocysts. There was no interaction between medium and protein source.

Ⅳ. DISCUSSION

The result of the present study shows that the initial cleavage of bovine IVF oocytes was affected by the supplemented protein source. The result also suggests that the *in vitro* development of bovine IVF embryos is affected by the culture medium and supplemented protein sources when the embryos are co-cultured with somatic cells.

In bovine, TCM-199 supplemented with 10% FBS was generally used for co-culture of IVF

Table 5. *In vitro* development of IVF bovine embryos co-cultured with Buffalo rat liver cells (BRLC) in TCM-199 or CR1aa supplemented with different protein sources

Medium	Protein sources	No. of embryos cultured*	%(mean±SE) of embryos developed to		
			Morula	Blastocyst	M+Bl**
TCM-199	BSA	103	12.2±2.7	30.5±2.6^{a}	42.6±0.7^{a}
	FBS	106	11.9±3.7	40.9±2.4^{bc}	58.7±2.9^{b}
CR1	BSA	103	10.6±2.2	37.4±1.8^{b}	48.0±2.0^{c}
	FBS	108	13.0±1.9	44.4±1.4^{c}	57.4±1.5^{b}

Total of 4 replicates.

* Two- to 8-cell stage embryos were used for *in vitro* culture.

** M: morula, Bl: blastocyst

^{a,b,c}Values within columns with different superscripts differ($P<0.05$).

embryos with somatic cells. In this study, however, BSA lather than FBS was more effective for the initial cleavage of the IVF embryos. When the IVF COCs were cultured in medium supplemented with FBS, cumulus cells formed a monolayer and pressed down the embryos to the bottom of dish, which might inhibiting the cleavage of embryos. Contrarily, in medium supplemented with BSA cumulus cells did not form a monolayer and developed in some masses without press the embryos. On the other hand, Fukui et al.(1996) cultured the bovine IVM oocytes in a modified Tyrode's fertilization medium containing BSA for 30 hr to allow the in vitro fertilization and initial cleavage.

CR1aa was more benefit for the blastocyst formation of bovine IVF embryos than a complex medium, TCM-199, when embryos were cultured without somatic cells. It was suggested that a certain factor contained in TCM-199 developed originally for tissue culture could inhibit the embryo development. Vitamins(Rosenkrans and first, 1991) and high concentration of glucose(Takahashi and First, 1992; Rorie et al., 1994; Edwards et al., 1997) could inhibit the development of bovine embryos.

Developmental potential of bovine embryos was improved by co-culture with somatic cells. It was suggested that such somatic cells may produce unknown, embryo trophic factors and/or inactivation the embryo toxic substances in the medium(Eyestone and First, 1989; Bavister et al., 1992). Bovine granulosa cells and BOEC produced a metalloproteinase-1, an embryogenesis-stimulating activity(Satoh et al., 1994)). Also, BOEC and BRLC modified the carbohydrate composition of the media by increasing the levels of L-lactate and pyruvate, and reducing glucose concentration(Edwards et al., 1997).

In co-culture system, supplemented protein sources affected the morphologies of co-culture cells. Cumulus cells formed a monolayer in medium supplemented with FBS, whereas formed suspended clusters in medium containing BSA. BOEC vesicles also formed a monolayer in medium containing FBS at 5 to 7 days after, but not in medium containing BSA. It was difficult to conclude that the development of bovine IVF embryos was affected by the morphology of co-culture cells because the development of embryos was not affected by the morphologies of CC and BOEC in CR1aa. It can be suggested that the BSA enhanced the ability of CC and BOEC to inactivate the embryo toxic substance in TCM-199.

BRLC was generally employed with a monolayer type in co-culture system(Rehman et al., 1994a,b; van Inzen et al., 1995). However, BRLC monolayer gradually dissociated from the bottom of culture dish and then degenerated in medium containing BSA, which might affect the development of embryos. On the other hand, BRLC monolayer supported embryo development in TCM-199 containing FBS. BRLC was significantly different from the other co-culture cells in a reduction of the O_2 tension and pH, and in change of the concentration of amino acids in TCM-199 containing FBS(Edwards et al., 1997). We could not, however, conclude that this was the reason of improved embryo development in TCM-199 containing FBS. Further studies are needed to determine the role of the BRLC on the development of bovine embryos in TCM-199 containing FBS.

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요 약

각종 공동배양 배지와 첨가 단백질원의 조합이 소 체외수정란의 체외배양에 미치는 영향

정희태 · 이준희 · 박춘근 · 양부근 · 김정익

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본 연구는 소 체외수정란의 체외배양 시 공동배양배지 및 첨가되는 단백질원에 따른 소 체외수정란의 체외 발육능을 검토하였다. 소의 미성숙 난포란을 체외에서 성숙, 수정시킨 후, BSA 또는 FBS를 첨가한 TCM-199 또는 CR1aa 배양액으로 단순배양 또는 난구세포, 소 난관상피세포(BOEC) 및 Buffalo rat 간세포(BRLC)와의 공동배양 후, 체외수정란의 분할율 및 발육능을 검사하였다. 소 성숙 난포란을 체외수정 후, 분할율은 배양액의 종류에 관계없이 BSA를 첨가한 경우에 유의적으로 높았다($P<0.01$). 분할된 수정란을 BSA 또는 FBS가 첨가된 TCM-199 또는 CR1aa 배양액 내에서 단순배양한 결과, 배반포 발육율은 단백질원에 관계없이 CR1aa 액에서 배양한 경우가 유의적으로 높았다($P<0.05$). 분할된 수정란을 난구세포 또는 BOEC와 공동배양 시, TCM-199 배양액에서는 FBS에 비하여 BSA 첨가구가 높은 배반포 형성율을 보였으나($P<0.05$), CR1aa 배양액에서는 BSA와 FBS 첨가구 모두 높은 발육율이 얻어졌다. 한편, 분할된 수정란을 BRLC의 단층세포와 공동배양 시에는 배양액의 종류와 관계없이 BSA에 비하여 FBS가 수정란의 발육율을 향상시켰다($P<0.05$). 본 실험의 결과는 배양액 중에 BSA첨가가 소 체외수정란의 분할을 촉진할 수 있으며, 체외수정란을 체세포와 공동배양 시, 수정란의 발육율이 배양액 및 첨가 단백질원의 종류에 따라 영향을 받아, TCM-199액에서 난구세포 또는 BOEC와 공동 배양하는 경우에는 BSA첨가가 효과적일 수 있음을 수 있음을 보여준다.

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